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**Title:** Targeting C3a/C5a Receptors Inhibits Human Mesangial Cell Proliferation and Alleviates IgA Nephropathy in Mice

**Short title:** Targeting C3a/C5a Receptors Alleviates IgA Nephropathy

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**Keywords:** complement, C3a receptor, C5a receptor, IgA nephropathy

**All abbreviations used:** IgA nephropathy, IgAN; C3a receptor, C3aR; C5a receptor, C5aR; human mesangial cells, HMCs; real-time quantitative polymerase chain reaction, RT-qPCR; wild-type, WT; transforming growth factor, TGF; tumour necrosis factor, TNF; interleukin, IL; monocyte chemotactic protein 1, MCP-1; blood urea nitrogen, BUN; serum creatinine, SCr; renin-angiotensin system, RAS; Kidney Disease Improving Global Outcomes, KDIGO; membrane attack complex, MAC; 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; dimethyl sulfoxide, DMSO; phosphate buffer saline, PBS; horseradish peroxidase, HRP; electrochemiluminescence, ECL; haematoxylin and eosin, H&E; periodic acid Schiff, PAS; one-way analysis of variance, ANOVA; C3a receptor antagonist, C3aRA; C5a receptor antagonist, C5aRA; antigen presenting cells, APCs

## Summary

Complement activation has a deep pathogenic influence in IgA nephropathy (IgAN). C3a and C5a, small cleavage fragments generated by complement activation, are key mediators of inflammation. The fragments exert broad pro-inflammatory effects by binding to specific receptors (C3aR and C5aR, respectively). However, no studies thus far have investigated the effects of C3a, C5a and their receptors on IgAN. We observed that C3aR and C5aR antagonists repressed IgA-induced cell proliferation and IL-6 and MCP-1 production in cultured human mesangial cells (HMCs). Furthermore, an IgAN mouse model induced by Sendai virus infection was employed to investigate the effects of C3aR and C5aR on IgAN *in vivo* for the first time. Wild-type (WT) and several knockout mouse strains (C3aR<sup>-/-</sup> or C5aR<sup>-/-</sup>) were immunised intranasally with increasing doses of inactivated virus for 14 weeks and were subjected to two intravenous viral challenges during the indicated time period. In the Sendai virus-induced IgAN model, C3aR/C5aR-deficient mice had significantly reduced proteinuria, lower renal IgA and C3 deposition, less histologic damage and reduced mesangial proliferation compared with WT mice. Both C3aR deficiency and C5aR deficiency, especially C3aR deficiency, significantly inhibited renal TNF- $\alpha$ , TGF- $\beta$ , IL-1 $\beta$ , IL-6 and MCP-1 expression. However, C3aR/C5aR-deficient and WT mice with IgAN did not differ with respect to their BUN and SCr levels. Our findings provide further support for the idea that C3aR and C5aR are crucially important in IgAN and suggest that pharmaceutically targeting C3aR/C5aR may hold promise for the treatment of IgAN.

## **Introduction**

IgA nephropathy (IgAN), characterised by the deposition predominantly of IgA in the mesangium, is the most prevalent primary glomerulonephritis worldwide. Although IgAN was previously considered a benign disease, researchers have recognised that up to 40% of IgAN patients will progress to ESRD within 20 years of diagnosis [1]. Various approaches intended to prevent IgAN progression have been attempted, but the best therapy for the disease remains incompletely established. According to the current Kidney Disease Improving Global Outcomes (KDIGO) Clinical Practice Guideline [2], renin-angiotensin system (RAS) inhibitors are recommended for controlling blood pressure and partly effective to achieve proteinuria <1g/d. Glucocorticoids are recommended for IgAN patients with persistent proteinuria. However, the broad severe side effects profile of steroids limits their use in patients, and evidence is still lacking regarding the efficacy of combined immunosuppressive therapy in patients with IgAN. There are no disease-targeted treatments for IgAN. The development of such treatments depends on an evolving understanding of the pathogenesis of the disease.

Accumulating evidence suggests that complement activation plays a prominent role in the pathogenesis of IgAN [3,4]. Activation of the complement system results in cleavage of C3 and C5, as well as generation of terminal effective products that have diverse biological functions, including induction of inflammatory responses by the small fragments (C3a, C5a), direct killing of pathogens by the membrane attack complex (MAC) C5b-9 and opsonisation of pathogens through C3b and its metabolites. C3a and C5a have gained more and more attention for their role in inducing overwhelming inflammation. By signalling through their G protein-coupled receptors (C3aR and C5aR or C5L2), C3a and C5a induce cytokine and chemokine release, neutrophil and macrophage

chemoattraction, and macrophage activation [5]. Previous studies have reported increased plasma concentrations of C3a [6,7], as well as glomerular overexpression of C5aR, in IgAN patients [8], findings indicative of the existence of a relationship between C3a and C5a and IgAN. Our previous study demonstrated that increased generation of C3a and C5a, as well as enhanced expression of their receptors, may be crucial for the progression of IgAN [9].

Therefore, inhibition of C3a, C5a and their receptors may be a promising therapy for the disease. Recently, two patients with rapidly progressive IgAN that were treated with an anti-C5 antibody (eculizumab) experienced renal functional improvement or stabilisation and reduced proteinuria [10,11]. These results highlight the need for further research exploring the potential usefulness of targeting C3a, C5a and their receptors as a therapy for the disease. The purpose of this study is to define the effect of C3aR/C5aR inhibition on IgA1-stimulating HMCs *in vitro* and to investigate whether C3a/C5a receptor deficiency can reduce the extent and severity of renal injury further in an IgAN mice model and thus provide a foundation for the development of a novel targeted therapy for IgAN in the future. An experimental model of IgAN induced by Sendai virus was employed here, because it mimicked the clinical character of IgAN, that a mucosal infection was always plays in evolution of the disease.

## **Materials and Methods**

### **HMCs culture**

A well-characterized human mesangial cell line was generously provided to us by Shanghai Medical College, Fudan University. The cells were cultivated in RPMI 1640 (Gibco, Grand Island, NY, USA)

medium supplemented with 10% foetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air.

#### HMC proliferation assay by MTT method *in vitro*

Cell proliferation was determined using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HMCs were grown to confluence, harvested by trypsinisation and seeded into a 96-well plate at a density of 10<sup>4</sup> cells/well. IgA1 isolated from pooled human serum was purchased from Sigma-Aldrich (St. Louis, MO, USA). Both the C3aR antagonist (Catalogue No. 559410, CAS 1140525-25-2) and the C5aR antagonist (Catalogue No. 234415, CAS 405098-33-1) were purchased from Calbiochem (San Diego, CA, USA). The cells were treated with blank medium or medium containing 100 µg/ml IgA, 100 µg/ml IgA+100 µM C3aR antagonist, or 100 µg/ml IgA+100 µM C5aR antagonist for 48 hr at 37°C. There were 6 parallel wells in every group and negative control group. After treatment, the cells were washed with serum-free medium, and 20 µl of 5 mg/ml MTT solution (Sigma, St. Louis, MO, USA) was added to each well. After incubation for 4 hr at 37°C, the MTT-containing medium was removed by aspiration. The blue formazan product generated was dissolved by the addition of 200 µl of 100% dimethyl sulfoxide (DMSO) to each well. The absorbance of solubilised blue formazan was recorded at 490 nm by a microplate reader. Cell proliferation percentages were calculated using the following equation: (mean OD of treated cells/mean OD of control cells) × 100.

#### Detection of receptor and cytokine mRNA expression by RT-qPCR

Total RNA was extracted using Trizol reagent (Invitrogen, Karlsruhe, Germany). Gel electrophoresis was used to determine RNA integrity. For real-time quantitative PCR, 5 µl of DNase-treated total RNA

was reverse-transcribed using an AMV First Strand cDNA Synthesis Kit. RT-qPCR was performed using an ABI StepOnePlus™ real-time fluorescence quantitative PCR instrument and ABI SybrGreen PCR Master Mix. PCR was set up in 96-well microplates containing 10 µl of Master Mix, 2 µl of cDNA, and 10 pmol of each 3' and 5' primer pair for either the target genes or the GAPDH gene (internal reference), yielding a 20-µl reaction volume. PCR amplification was carried out for 3 min at 95°C and for 20 s at 60°C, followed 40 cycles of 20 s at 72°C. Gene expression was expressed as  $2^{-\Delta\Delta Ct}$ , where Ct is the cycle threshold,  $\Delta Ct = Ct (\text{target gene}) - Ct (\text{GAPDH})$ , and  $\Delta\Delta Ct = \Delta Ct (\text{IgAN}) - \Delta Ct$  (normal control). The primer sequences are shown in Table 1.

**Table 1. Primers used in transcription–polymerase chain reaction**

Genes	Primer 1 (Forward)	Primer 1 (Reverse)
Human C3aR	5'-CCCACTGTCCCTCAAACAAT-3'	5'-AAGTCCGCTGCTCACCATA-3'
Human C5aR	5'-GGCTGTCTTTCTTGTCTGCTG-3'	5'-CAAATCGTGAGCGTGAGTAGAG-3'
Human IL-6	5'-AAGCAGCAAAGAGGCACTG-3'	5'-ATGATTTTCACCAGGCAAGTCT-3'
Human MCP-1	5'-GCTGAGACTAACCCAGAAACATC-3'	5'-GAATGAAGGTGGCTGCTATGAG-3'
Human GAPDH	5'-TGGGTGTGAACCATGAGAAGT-3'	5'-TGAGTCCTTCCACGATACCAA-3'
Mouse TNF- $\alpha$	5'-CACCACCATCAAGGACTCAA-3'	5'-GAGACAGAGGCAACCTGACC-3'
Mouse TGF- $\beta$	5'-CCGCAACAACGCCATCTAT-3'	5'-CCAAGGTAACGCCAGGAATT-3'
Mouse IL-1 $\beta$	5'-GGGAAACAACAGTGGTCAGGA-3'	5'-TGCTCATTACGAAAAGGGA-3'
Mouse IL-6	5'-AGGAGTGGCTAAGGACCAAGAC-3'	5'-AGTGAGGAATGTCCACAAACTGATA-3'
Mouse MCP-1	5'-TCTCTCTTCCTCCACCACCAT-3'	5'-GCTCTCCAGCCTACTCATTGG-3'
Mouse GAPDH	5'-GGTGAAGGTCGGTGTGAACG-3'	5'-CTCGCTCCTGGAAGATGGTG-3'

C3aR: C3a receptor; C5aR: C5a receptor; TNF: tumour necrosis factor; TGF: transforming growth factor; IL: interleukin; MCP-1: monocyte chemotactic protein 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

#### Detection of receptor and cytokine protein expression by Western blotting

The cells were washed with ice-cold phosphate buffer saline (PBS) and gently resuspended in ice-cold RIPA buffer with freshly added 1% protease inhibitor cocktail before being incubated on ice for 10 min. The cell lysates were centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant was prepared as a protein extract. The protein concentration was determined using a bicinchoninic acid assay kit

(Dingguo Bioscience, Beijing, China). Samples containing 60 µg of total protein were separated on a 10% sodium dodecylsulfate polyacrylamide gel and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk solution in 0.1% tris-buffered saline Tween-20 (Dingguo Bioscience, Beijing, China) for 1 hr. The membranes were subsequently incubated with either mouse anti-human C3aR monoclonal antibodies (1:500 dilution, AbD Serotec, Oxford, UK), mouse anti-human C5aR monoclonal antibodies (1:500 dilution, AbD Serotec, Oxford, UK), rabbit anti-human IL-6 monoclonal antibodies (1:800 dilution, Abcam, Cambridge, UK), rabbit anti-human MCP-1 polyclonal antibodies (1:1000 dilution, Abcam, Cambridge, UK) or rabbit anti-human GAPDH polyclonal antibodies (1:800 dilution, Good Here Bioscience, Hangzhou, China) overnight at 4°C with gentle shaking. After washing, HRP-conjugated secondary antibodies (1:2000 dilution, Jackson Immuno-Research, West Grove, PA, USA) were added to the mixture, which was incubated for 2 hr at 37°C. Antibody binding was detected using an electrochemiluminescence (ECL) detection kit (Thermo Scientific, Rockford, IL, USA) to produce a chemiluminescence signal, which was captured on X-ray film. Band intensities were quantified from scanned membrane images using Image J software (National Institutes of Health, Bethesda, MD, USA).

## Animals

Homozygous C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> mice on BALB/c genetic background were purchased from Jackson Laboratory (Stock No.: 005712 and 006845, respectively; Jackson Labs, Bar Harbor, ME, USA). WT BALB/c mice were purchased from the Laboratory Animal Centre of Zhengzhou University. Female mice (6-8 weeks old) were used for model preparation. The animal procedures were performed in



accordance with the institution's guidelines for the care and use of laboratory animals and were approved by the ethics committee of our university.

#### Sendai virus preparation

Sendai virus Strain52 was obtained from the Wuhan Institute of Virology, Chinese Academy of Sciences. Expansion of infectious suspensions of viral stock was conducted in chicken embryos. A total of 0.2 ml of Sendai virus concentrate was diluted in 1 ml of virus suspension with PBS, and the virus dilution was injected into live fertile eggs (0.15 ml per egg) at a point opposite the embryo. The eggs were placed in an incubator at 37°C. Live eggs were selected by “candling” after 24 hr, and then these eggs were incubated further at 37°C for 28 hr. After 52 hr of incubation, the eggs were chilled to 4°C and incubated overnight. Allantoic fluid was withdrawn from the eggs slowly and then centrifuged at 3000 r/min at 4°C for 25 min. The supernatant was divided into 1-ml working stock aliquots and stored in liquid nitrogen for use. The virus titre for each mouse was calculated as the geometric mean of the number of plaques formed in a haemagglutination plaque assay, multiplied by the dilution factor.

#### Induction and analysis of the mouse IgAN model

The mouse IgAN model was generated by Sendai virus infection, as previously described [12,13]. Three groups of each mouse strain (C3aR<sup>-/-</sup>, C5aR<sup>-/-</sup> and WT, seven mice per group) were immunised intranasally with 0.2 ml of a suspension containing inactivated Sendai virus weekly for fourteen weeks. The initial dose of inactivated virus was  $2.5 \times 10^4$  for the first and second times to reduce morbidity and mortality from viral pneumonia. Subsequently, the amount of inactivated virus administered to the immunised mice gradually increased to  $2.5 \times 10^5$  at the third week,  $2.4 \times 10^6$  at the fourth week and

$1.92 \times 10^8$  from week 5 to 14. On week 6 and 14, the immunised mice were challenged intravenously with  $6.25 \times 10^8$  of virus via the tail vein. Seven WT mice that were not exposed to any virus served as negative controls and received an equivalent volume of sterile PBS on the same days and by the same route as the immunised groups. Uninfected and Sendai virus-infected experimental mice were housed in microisolator cages in separate rooms, were isolated from each other and from the other animals, and were fed *ad libitum* with sterile food and water.

24 hr urine samples were collected from each mouse in metabolic cages before immunisation and at the end of the fourteenth week for proteinuria analysis. Urinary protein was measured with a pyrogallol red-molybdate complex in an automated analyser (CS-400B; Dirui, Changchun, China). Blood samples (0.2-0.3 ml per mouse) were obtained by puncturing the retrobulbar veins with capillary glass pipettes one day before the experiment. Blood samples (0.8-1.2 ml per mouse) were again taken from the retrobulbar veins after sacrifice. Renal function was assessed by measuring SCr and BUN concentrations on an automatic biochemical analyser (CS400; Roche, Rotkreuz, Switzerland). Serum C3 level determinations were performed using enzyme-linked immunosorbent assay (ELISA) kits (Abcam, Cambridge, UK), according to the manufacturer's instructions.

#### Histologic analysis

The mice were sacrificed at the end of week 14. The mice were anaesthetised with 10% chloral hydrate. Laparotomy was performed, and the kidneys were harvested. One-fourth of one kidney was fixed in 4% paraformaldehyde (pH 7.4) and embedded in paraffin. The paraffin sections (3  $\mu$ m) were stained in haematoxylin and eosin (H&E), periodic acid Schiff (PAS) and Masson trichrome. Evaluation of renal histologic changes was performed blindly by two pathologists using a light microscope. A sample of

fresh renal cortex (approximately 1/8 of the kidney) was optimal cutting temperature-embedded, snap-frozen, and sectioned at 4  $\mu$ m. For direct immunofluorescence microscopy, slides were incubated with either fluorescein isothiocyanate-conjugated goat anti-mouse IgA polyclonal antibodies (1:10 dilution, AbD Serotec, Oxford, UK) or rat anti-mouse C3 monoclonal antibodies (1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at 37°C. After washing, slides were observed with a fluorescence microscope. Staining intensity was expressed as follows: negative, invisible; weak, ambiguous under a low-power lens and apparent under a high-power lens; moderate, apparent under a low-power lens and clear under a high-power lens; intense, clear under a low-power lens and bright under a high-power lens; and very intense, dazzling under a high-power lens. The remaining kidney tissues were stored in liquid nitrogen for use.

#### RNA extraction from kidney tissues

About 50mg of thawed kidney tissue samples was homogenized in 1 ml of Trizol reagent (Invitrogen, Karlsruhe, Germany). The homogenized samples were then incubated for 10 min at room temperature to permit complete dissociation of RNA. 0.2 ml of Chloroform was added to the homogenized samples, which were shaken vigorously and incubated again at room temperature for 5 min. The samples were centrifuged at 12,000 r/min at 4°C for 20 min, and the aqueous phase was transferred to a new tube. 0.5 ml of Isopropyl alcohol was added to the aqueous phase to precipitate the RNA. Samples were mixed and incubated at room temperature for 10 min and then centrifuged at 12,000 r/min at 4°C for 15min. The supernatant was removed and the RNA pellet was washed with 1ml of 70% ethanol. The sample was again centrifuged at 12,000 r/min at 4°C for 3 min. After discarding the supernatant, the pellet was air-dried, after which it was resuspended in 100  $\mu$ l of RNase-free water. The RNA samples were treated

with DNase, and then purified on RNeasy Mini columns (Qiagen, Valencia, CA, USA) and washed out with 50 µl of RNase-free water. Renal TNF- $\alpha$ , TGF- $\beta$ , IL-1 $\beta$ , IL-6 and MCP-1 mRNA expression levels were detected by RT-qPCR as mentioned above.

#### Detection of renal cytokine expression by immunohistochemistry

The paraffin-embedded sections were cut, deparaffinised, and hydrated by soaking in 100% xylene and sequential descending ethanol concentrations, followed by microwave heating in citrate buffer (pH 6.0). Next, the sections were incubated in 0.3% H<sub>2</sub>O<sub>2</sub> in PBS to block endogenous peroxidase activity. After washing, the sections were incubated with either anti-TNF- $\alpha$  polyclonal antibodies (1:200 dilution, Abcam, Cambridge, UK), anti-TGF- $\beta$  polyclonal antibodies (1:200 dilution, Abcam, Cambridge, UK), anti-IL-1 $\beta$  polyclonal antibodies (1:400 dilution, Bioss, Beijing, China), anti-IL-6 polyclonal antibodies (1:400 dilution, Bioss, Beijing, China) or anti-MCP-1 polyclonal antibodies (1:300 dilution, Abcam, Cambridge, UK) overnight at 4°C in a moist chamber. Biotinylated secondary antibodies (1:100 dilution, Zhongshan Golden Bridge, Beijing, China) were incubated for 20 min at 37°C, and avidin-biotin-peroxidase complexes (1:20 dilution, Zhongshan Golden Bridge, Beijing, China) were applied. Colour development was obtained by treatment with diaminobenzidine. Finally, the sections were counter-stained with haematoxylin, dehydrated, sealed and observed by light microscopy. The positively stained cells in 10 fields of 2 stained kidney sections from each animal were quantified at a magnification of  $\times 200$  and expressed as numbers of cells per field.

#### Statistical analysis

Data are presented as the mean  $\pm$  SD. Significant differences between multiple groups were analysed

by one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. Paired-sample *t*-tests were used to compare proteinuria and renal function before and after 14 weeks of Sendai virus immunisation, and the immunofluorescence results for IgA and C3 were compared by the Mann-Whitney U test. A *P* value less than 0.05 was considered statistically significant. All analyses were performed using the SPSS statistical software package, version 17.0 (SPSS Inc., Chicago, IL, USA).

## Results

IgA stimulation increased C3aR/C5aR expression in HMCs

RT-qPCR and Western blot analysis demonstrated increased C3aR and C5aR expression in HMCs incubated with 100 µg/L IgA for 48 hr, findings demonstrative of C3aR and C5aR activation in HMCs after IgA stimulation. Elevated C3aR or C5aR expression could be inhibited by C3aR or C5aR antagonism, respectively.

C3aR/C5aR antagonism inhibited HMC proliferation after IgA stimulation *in vitro*

Enhanced cell proliferation, as measured by MTT assay, was observed when HMCs were incubated with 100 µg/L IgA for 48 hr (cell viability: 168.1±5.0% *versus* 100.0±6.9%, in the IgA-stimulated and blank control groups, respectively, *P* < 0.001; Figure 1a). We subsequently investigated the potential effects of C3aR or C5aR antagonism on modulating IgA-stimulated HMC proliferation. Administration of a C3aR or C5aR antagonist at a concentration of 100 µM significantly inhibited HMC proliferation after IgA stimulation (cell viability in the IgA-stimulated+C3aR antagonist group: 113.8±9.3%, cell viability in the IgA-stimulated+C5aR antagonist group: 121.5±4.6%, *P* < 0.001 *versus* the

IgA-stimulated group; Figure 1a). There was no significant difference in the HMC proliferation down-regulation elicited by C3aR *versus* C5aR antagonism ( $P = 0.363$ ).

#### C3aR/C5aR antagonism reduced IL-6 and MCP-1 up-regulation after IgA stimulation *in vitro*

Elevated IL-6 and MCP-1 mRNA expression was observed when the HMCs were incubated with IgA-containing medium, and these elevations were significantly reduced by the addition of a C3aR or C5aR antagonist (Figure 1b). Correspondingly, IL-6 and MCP-1 protein synthesis was up-regulated in all the IgA-stimulated groups compared with the negative control group (Figure 1c, 1d). IL-6 and MCP-1 expression up-regulation was reduced by C3aR or C5aR antagonism (Figure 1c, 1d), paralleling our findings regarding gene expression. With respect to IL-6 and MCP-1 gene expression and protein synthesis levels, no differences between C3aR antagonism and C5aR antagonism were noted ( $P > 0.05$ ).

#### Sendai virus infection induced IgAN in mice

Proteinuria was considered evidence of glomerular dysfunction and clinical nephritis. After 14 weeks of immunisation, proteinuria was exhibited by immunised WT mice, whose daily proteinuria increased from  $1.38 \pm 0.35$  mg to  $2.63 \pm 0.39$  mg ( $P = 0.001$ ). Proteinuria levels in immunised WT mice were significantly greater than those in non-immunised controls at the end of week 14 ( $2.63 \pm 0.39$  mg/24 h *versus*  $1.12 \pm 0.25$  mg/24 h,  $P < 0.001$ ). IgA and C3 deposition was not observed in the normal glomeruli of non-immunised mouse renal specimens. Granular-positive staining of IgA accompanied by C3 deposition was noted in the mesangium of glomeruli of immunised WT mice at week 14, a finding demonstrative of the diagnosis of IgAN after immunisation.

### C3aR/C5aR deficiency reduced proteinuria in IgAN mice

Proteinuria in C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> IgAN mice was significantly reduced compared to that in WT mice with IgAN (1.89±0.46 g/24 h in C3aR<sup>-/-</sup> IgAN mice *versus* 2.63±0.39 g/24 h in WT IgAN mice, *P* = 0.005; 1.59±0.31 g/24 h in C5aR<sup>-/-</sup> IgAN mice *versus* 2.63±0.39 g/24 h in WT IgAN mice, *P* < 0.001; Table 2). No significant difference in serum creatinine or BUN was detected among the various groups, including the control group (*P* > 0.05). Serum C3 levels in C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> IgAN mice were significantly lower than those in WT IgAN mice (*P* = 0.017 and 0.049, respectively).

**Table 2. Renal function and proteinuria in immunised and non-immunised mice**

Groups	BUN (mmol/L)		SCr (μmol/L)		Proteinuria (g/24 h)	
	Day -1	Week 14	Day -1	Week 14	Day -1	Week 14
Non-immunised WT mice	4.29±0.66	4.37±0.62	21.09±1.11	23.09±4.27	1.02±0.21	1.12±0.25
Immunised WT mice	4.59±0.53	4.73±0.56	21.46±1.24	23.31±2.79	1.38±0.35	2.63±0.39*
Immunised C3aR <sup>-/-</sup> mice	4.53±0.43	4.96±0.68	21.40±1.30	23.23±3.62	1.27±0.27	1.89±0.46* <sup>#</sup>
Immunised C5aR <sup>-/-</sup> mice	4.50±0.37	4.71±0.65	21.30±1.33	23.59±4.93	0.98±0.30	1.59±0.31* <sup>#</sup>

Data are expressed as the mean ± standard deviation. \**P* < 0.05 before and after 14 weeks of Sendai virus immunisation. <sup>#</sup>*P* < 0.05 *versus* immunised WT mice at week 14. BUN: blood urea nitrogen; SCr: serum creatinine.

### C3aR/C5aR deficiency markedly attenuated histologic injury

No IgA and C3 staining was noted in normal renal tissues. Immunofluorescence revealed IgA deposits accompanied by C3 deposits in the mesangium of the glomeruli from Sendai virus-immunised mice at week 14. Less IgA deposition was observed in the mesangium of C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> IgAN mice than in the mesangium of WT IgAN mice (*P* = 0.011 and 0.007, respectively; Figure 2a, Table 3). C3 staining intensity in the mesangium was also reduced in C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> IgAN mice compared with WT IgAN mice (*P* = 0.033; Figure 2b, Table 3). Light micrographs of the kidney tissues from immunised WT mice showed mesangial matrix expansion and hypercellularity, as well as some interstitial infiltration. Mesangial matrix expansion and hypercellularity were also reduced in C3aR<sup>-/-</sup>

and C5aR<sup>-/-</sup> IgAN mice (Figure 2c).

**Table 3. Glomerular IgA and C3 deposition in the renal biopsies from each group of mice**

Groups	IgA intensity ( <i>n</i> )					C3 intensity ( <i>n</i> )				
	Negative	Weak	Moderate	Intense	Very intense	Negative	Weak	Moderate	Intense	Very intense
Non-immunised WT mice	7	0	0	0	0	7	0	0	0	0
Immunised WT mice	0	0	3	3	1	1	0	3	3	0
Immunised C3aR <sup>-/-</sup> mice	1	2	4	0	0	3	2	2	0	0
Immunised C5aR <sup>-/-</sup> mice	1	3	3	0	0	3	2	2	0	0

Glomerular IgA and C3 deposition was measured by immunofluorescence staining. The numbers in the table cells represent the numbers of mice with the corresponding immunofluorescence staining intensities. After being immunised with the Sendai virus to induce IgA nephropathy, C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> mice exhibited significantly reduced IgA and C3 deposition in the kidney compared to WT mice ( $P < 0.05$ ).

#### C3aR/C5aR deficiency reduced cytokine and chemokine up-regulation in IgAN mice

No positive staining of cytokines and chemokines, including TNF- $\alpha$ , TGF- $\beta$ , IL-1 $\beta$ , IL-6 and MCP-1, was noted in the normal glomeruli from the renal specimens of the negative control group. Deposition of these cytokines and chemokines was scarcely observed in the tubular epithelial cells of normal kidney tissues. For WT IgAN mouse renal specimens, IL-1 $\beta$  and MCP-1 were deposited mainly in tubular epithelial cells and occasionally in mesangial cells, while IL-6, TNF- $\alpha$  and TGF- $\beta$  were deposited mainly in mesangial cells and tubular epithelial cells. The deposits of all these factors in C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> IgAN mice had similar distribution patterns (Figure 3, Supplementary Figure S1). However, the number of TNF- $\alpha$ , TGF- $\beta$ , IL-1 $\beta$ , IL-6 or MCP-1 positive cells was significantly lower in C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> IgAN mice than in IgAN mice (Figure 3, Supplementary Figure S1, Table 4). In addition, the number of IL-1 $\beta$  or IL-6 positive cells was significantly lower in C3aR<sup>-/-</sup> IgAN mice than in C5aR<sup>-/-</sup> IgAN mice ( $P = 0.034$  and  $0.015$ , respectively), while the number of MCP-1 positive cells was marginally lower in the former group than in the latter ( $P = 0.057$ ). The numbers of TNF- $\alpha$  and



TGF- $\beta$  positive cells were comparable between C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> IgAN mice.

**Table 4. Cytokines and chemokine expression in the renal tissues from each group of mice**

Cytokines/ chemokines	Cells/Field			
	non-immunised WT mice	WT IgAN mice	C3aR <sup>-/-</sup> IgAN mice	C5aR <sup>-/-</sup> IgAN mice
TNF- $\alpha$	8.57 $\pm$ 2.82	24.14 $\pm$ 3.98 <sup>*</sup>	18.00 $\pm$ 4.43 <sup>* #</sup>	16.57 $\pm$ 4.04 <sup>* #</sup>
TGF- $\beta$	10.71 $\pm$ 2.56	38.00 $\pm$ 5.23 <sup>*</sup>	24.14 $\pm$ 5.79 <sup>* #</sup>	29.43 $\pm$ 6.08 <sup>* #</sup>
IL-1 $\beta$	1.29 $\pm$ 0.49	8.71 $\pm$ 1.98 <sup>*</sup>	4.14 $\pm$ 1.35 <sup>* #</sup>	6.43 $\pm$ 1.40 <sup>* #</sup>
IL-6	4.86 $\pm$ 1.57	29.57 $\pm$ 5.68 <sup>*</sup>	12.29 $\pm$ 4.31 <sup>#</sup>	21.14 $\pm$ 6.52 <sup>* #</sup>
MCP-1	6.57 $\pm$ 1.51	30.14 $\pm$ 6.39 <sup>*</sup>	15.86 $\pm$ 3.58 <sup>* #</sup>	20.43 $\pm$ 4.86 <sup>* #</sup>

Renal cytokine and chemokine expression was assessed by immunohistochemistry. The results were calculated as the numbers of positively stained cells quantified in 10 fields of 2 stained kidney sections from each animal at a magnification of  $\times 200$ . Data are expressed as the mean  $\pm$  standard deviation. <sup>\*</sup>*P* < 0.05 *versus* non-immunised WT mice. <sup>#</sup>*P* < 0.05 *versus* WT IgAN mice. C3aR: C3a receptor; C5aR: C5a receptor; TNF: tumour necrosis factor; TGF: transforming growth factor; IL: interleukin; MCP-1: monocyte chemotactic protein 1.

The RT-qPCR results showed that renal TNF- $\alpha$ , TGF- $\beta$ , IL-1 $\beta$ , IL-6 and MCP-1 mRNA expression levels were elevated in all three immunised groups compared with the negative control group. The gene expression levels of these cytokines and chemokines were significantly reduced in C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> IgAN mice compared with WT IgAN mice (Figure 4). In particular, renal IL-1 $\beta$ , IL-6 and MCP-1 mRNA were expressed in lower amounts in C3aR<sup>-/-</sup> IgAN mice than in C5aR<sup>-/-</sup> IgAN mice (Figure 4).

## Discussion

C3a and C5a are the major inflammatory effectors of complement activation and exert pro-inflammatory effects by binding to their respective receptors. In the current study, we found that C3aR/C5aR antagonists inhibited HMC proliferation induced by IgA1 stimulation *in vitro*. Furthermore, we demonstrated that C3aR or C5aR deficiency can also inhibit renal cytokine and

chemokine expression *in vivo*. Moreover, the current study is the first report to demonstrate that C3aR or C5aR deficiency can successfully reduce proteinuria and attenuate renal histologic injury in the IgAN mice model. Our results serve as definitive evidence indicating that C3a, C5a and their receptors play prominent roles in the pathogenesis of IgAN and represent a foundation on which potential therapies targeting and blocking C3aR/C5aR to treat IgAN in the future can be based.

Our data showed that C3aR/C5aR deficiency markedly reduced urinary protein levels in IgAN mice. However, no significant improvements in renal function were observed. However, administration of an anti-C5 antibody to a patient with rapidly progressive IgAN led to clinical improvements, namely, glomerular filtration rate stabilisation and reduced proteinuria [10]. Another case report also demonstrated a significant immediate improvement in renal function after anti-C5 antibody administration to patients with crescentic IgAN [11]. The diverse effects of complement inhibition on renal function may be attributed to differences in renal injury severity between the mouse model and the reported cases. The mouse model used in this study is a model of proliferative IgAN. No significant elevations in serum creatinine were noted after model preparation. However, both reported cases presented with more severe IgAN with renal insufficiency. Further research may be carried out on heminephrectomized HIGA mice, a potential model for progressive glomerulosclerosis of human IgAN [14], to clarify whether C3a/C5a blockage is useful for maintaining renal function. In addition, the diverse effects of complement inhibition on renal function may result from the different impacts of various effectors on the kidney. Anti-C5 antibodies can block MAC and C5a production, but not C3a production. In our previous study, C3a and C5a showed better correlations with proteinuria than renal function. In contrast, MAC was reported to be better correlated with renal function than proteinuria

[15]. These results indicate that the major effectors of complement activation, C3a, C5a and MAC, may play slightly different roles in the progression of the disease. Further research is necessary to clarify their precise roles in the pathogenesis of IgAN.

It is worth noting that IgA deposition was markedly decreased in the kidney of C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> mice compared to WT mice in the present IgAN model. However, conventional wisdom suggests that IgA deposits in the mesangium initially and then induces complement activation to generate terminal effectors, including C3a and C5a. It thus seems confusing IgA deposition was affected by C3aR/C5aR deficiency. This result strongly suggested that C3a/C5a activation took place earlier than IgA deposition in the kidney. Several studies have described increased plasma levels of C3 breakdown products in patients with IgAN [7,16,17], indicating that complement activation can take place on circulating IgA1-containing immune complexes before their deposition in the mesangium. Moreover, according to our data, C3a and C5a activation seems to prompt IgA deposition rather than occur as a sequential consequence of IgA deposition. The influence of C3a and C5a on B-cell responses has been known for many years, but the notion that C3a and C5a also contribute to the activation, expansion, and survival of T cells and antigen presenting cells (APCs) has only recently gained impetus [18]. C3a, C5a and their receptors are supposed to modify APC antigen presentation and participate in the formation of pathogenic immune complexes composed of galactose-deficient IgA1 and antiglycan antibodies [4]. If this is the case, the abovementioned decrease in IgA deposition in the kidney of C3aR/C5aR-deficient mice was reasonable. Future works will need to address the impact of anaphylatoxins on the production of antiglycan antibodies and the formation of pathogenic immune complexes.

In theory, inhibiting C3aR or C5aR should not affect the complement activation step since C3a and C5a are downstream effectors. However, we observed that C3 depletion in the circulation, as the result of systemic complement activation, was partly rescued by C3aR- or C5aR-deficiency in the IgAN mice model. C3aR or C5aR deficiency also decreased renal C3 deposition in the present IgAN mice model, indicating that local complement activation in the kidney was restrained. This suggested that complement activation may be regulated by a feedback loop. It has been reported that decreased joint C3 deposition occurred in C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> mice compared to WT mice in a collagen antibody-induced arthritis model [19], findings supporting the possible existence of a positive feedback pathway regulating C3 activation that is promoted by C3a and C5a through their receptors.

Our study found that a lack of C3aR or C5aR reduced renal expression of the pro-inflammatory factors TNF- $\alpha$ , TGF- $\beta$ , IL-1 $\beta$ , IL-6 and MCP-1 *in vivo* and may thus contribute to the amelioration of proteinuria and histologic injury in C3a/C5a-deficient IgAN mice. In addition, IL-1 $\beta$ , IL-6 and MCP-1 expression levels were significantly decreased in C3aR-deficient IgAN mice compared to C5aR-deficient IgAN mice. A recent study also found that C3aR-deficient dendritic cells produced less IL-1 $\beta$  [20], results that corresponded with ours. IL-1 $\beta$ , IL-6 and MCP-1 are generated by monocytes/macrophages and T cells. It was reported C3aR deficiency rather than C5aR deficiency led to significantly reduced monocyte/macrophage and T cell recruitment in the tubulointerstitium [21], providing a possible explanation for the abovementioned decreases in renal IL-1 $\beta$ , IL-6 and MCP-1 expression in C3aR-deficient IgAN mice.

Interestingly, C3aR deficiency, but not C5aR deficiency, significantly inhibited the renal expression of some cytokines and chemokines (such as IL-1 $\beta$ , IL-6 and MCP-1) *in vivo*; however, C3aR and C5aR deficiency seemed to have similar protective effects on proteinuria and histologic damage. Cytokine and chemokine release induced by C3a or C5a precedes renal damage and clinical presentations; thus, we suppose that discrepancies in cytokine and chemokine expression could be observed in earlier stages of the disease. Distinct roles for C3a and C5a were demonstrated in complement-induced tubulointerstitial injury [21]. In the indicated previous study, C3aR deficiency led to significantly reduced renal leukocyte infiltration and less severe tubulointerstitial inflammation and fibrosis and ultimately preserved renal function. In contrast, the absence of C5aR was inconsequential. The histologic changes in our mouse model were very slight, without obvious tubulointerstitial injury, so the diverse roles of C3a and C5a cannot be documented. However, the differences in the renal expression levels of the abovementioned cytokines and chemokines provided us with some clues that C3a/C3aR signalling may play a more important role in IgAN progression. Blocking C3aR may be a feasible treatment for severe IgAN.

Mesangial cells were found to secrete pro-inflammatory factors and proliferate after polymeric IgA stimulation in our study. This pathogenic course could be blocked by C3aR or C5aR deficiency, verifying the protective effects of C3aR/C5aR deficiency on mesangial cells *in vitro*. Remarkably, the reductions in IL-6 and MCP-1 secretion caused by C3aR or C5aR blockage were not significantly different in cultured mesangial cells, while C3aR deficiency demonstrated a stronger potential to inhibit IL-6 and MCP-1 renal expression than C5aR deficiency *in vivo*, perhaps because of the loss of inflammatory cell chemotaxis activity *in vitro*.

In conclusion, in this study, we demonstrated that C3aR/C5aR deficiency resulted in reduced proteinuria and attenuated histologic injury in an IgAN mouse model. This protective effect may be attributed partly to inhibition of the renal expression of cytokines and chemokines. We also validated that blocking C3aR or C5aR can inhibit cytokine and chemokine secretion and cell proliferation in cultured HMCs. Our data have provided us with profound evidence indicating that blocking C3aR or C5aR may be a promising therapy for patients with IgAN.

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### **Author contributions**

Guolan Xing conceived the study, designed the experiments and revised the article. Ying Zhang analysed the data, interpreted the results and drafted the article. Xianli Yan and Ting Zhao performed the experiments. Qihe Xu contributed significantly to the revision of the manuscript and to the final approval of the version to be published. Qi Peng contributed to the conception of the study. Ruimin Hu helped perform the analysis and prepare the manuscript. Yali Zhou and Songxia Quan participated in the histopathologic analysis.

### **Disclosures**

All authors declare no conflicts of interest.

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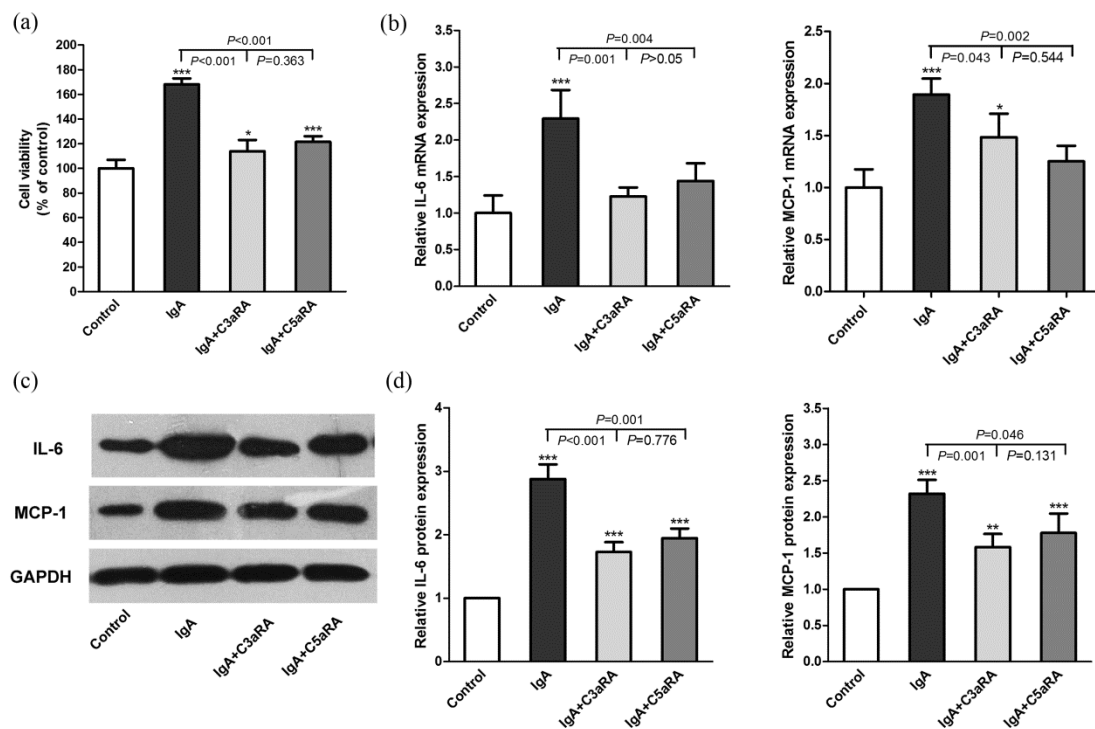


Fig. 1. Cell proliferation and cytokine mRNA and protein expression in cultured human mesangial cells (HMCs). The HMCs were treated with blank medium or medium containing 100  $\mu$ g/L IgA, 100  $\mu$ g/L IgA+100  $\mu$ M C3aR antagonist (C3aRA), or 100  $\mu$ g/L IgA+100  $\mu$ M C5aR antagonist (C5aRA) for 48 hr. The data are expressed as the mean  $\pm$  standard deviation (SD). (a) HMC proliferation was measured using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay ( $n = 6$  for each group). (b) IL-6 (left row) and MCP-1 (right row) gene expression levels relative to GAPDH were determined by RT-qPCR. (c) Western blot analysis of IL-6, MCP-1 and GAPDH protein expression. (d) Quantitative analysis for Western blot of IL-6 and MCP-1 protein expression. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  versus negative control.



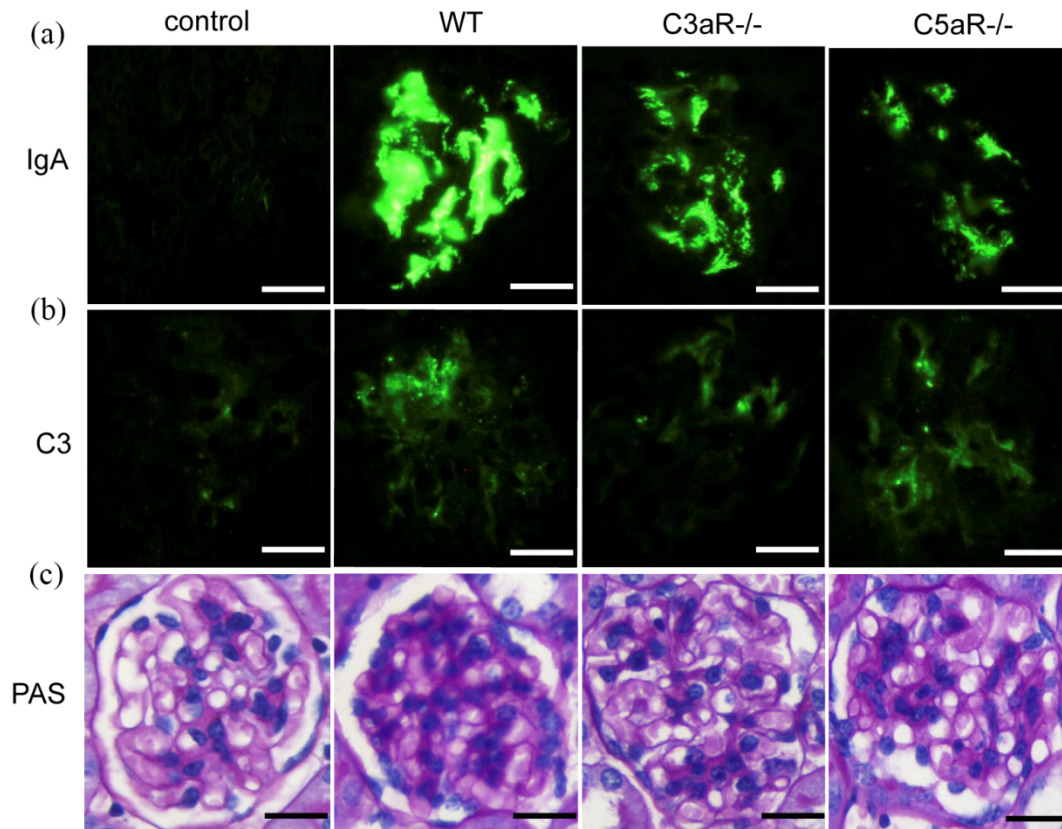


Fig. 2. Wild-type, C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> were immunised with inactivated Sendai virus to induce IgA nephropathy (IgAN). After 14 weeks of immunisation, IgA and C3 deposition in the mesangium and the associated histologic changes were analysed. (a-b) Glomerular IgA (upper row) and C3 (low row) deposition was measured by immunofluorescence staining of kidney sections from wild type (WT), C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> IgAN mice and negative controls. (c) Mesangial matrix expansion and cell proliferation were demonstrated by periodic acid-Schiff (PAS) staining of renal tissue in the following four groups of mice: negative controls and WT, C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> IgAN mice. Scale bars represent 100 μm.

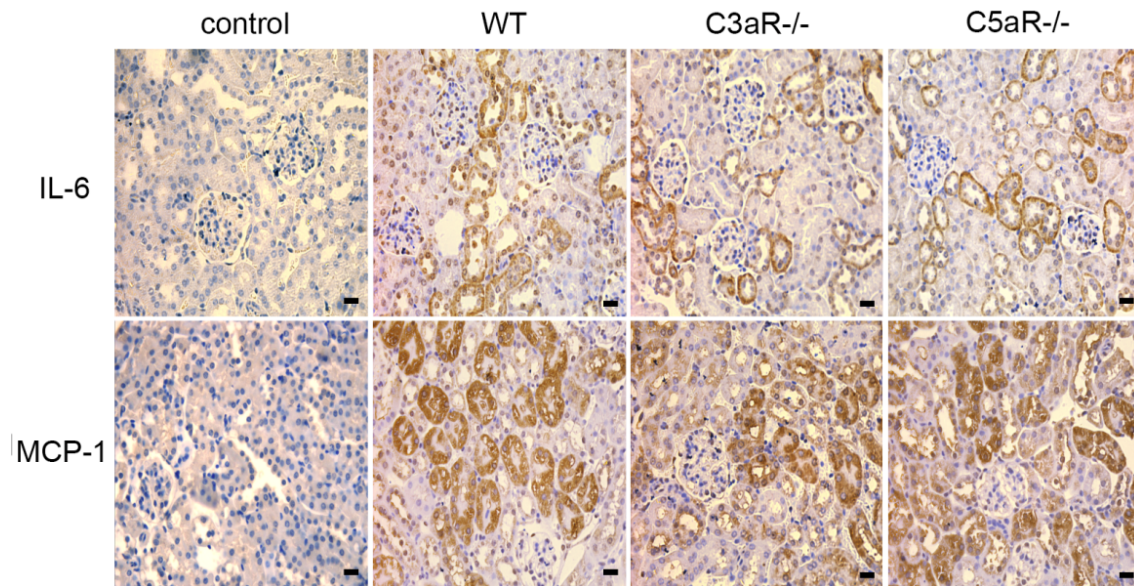


Fig. 3. Representative images of immunohistochemical staining for IL-6 and MCP-1 in the kidney sections of WT, C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> IgAN mice and negative controls. C3aR and C5aR deficiency reduced renal IL-6 and MCP-1 expression compared to WT mice. Scale bars represent 100  $\mu$ m.

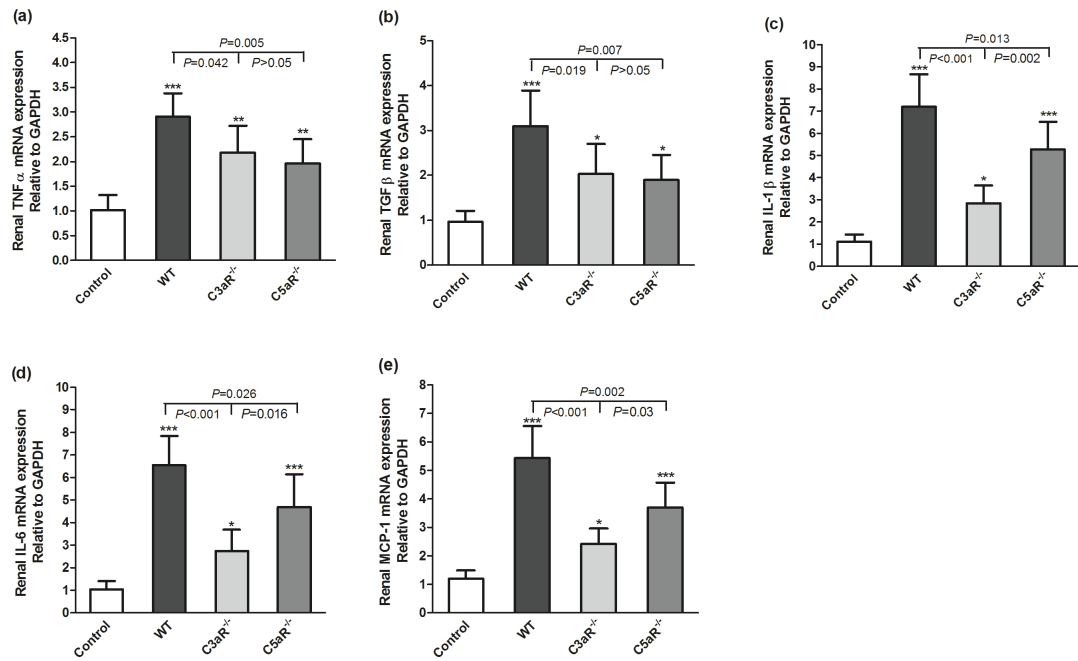
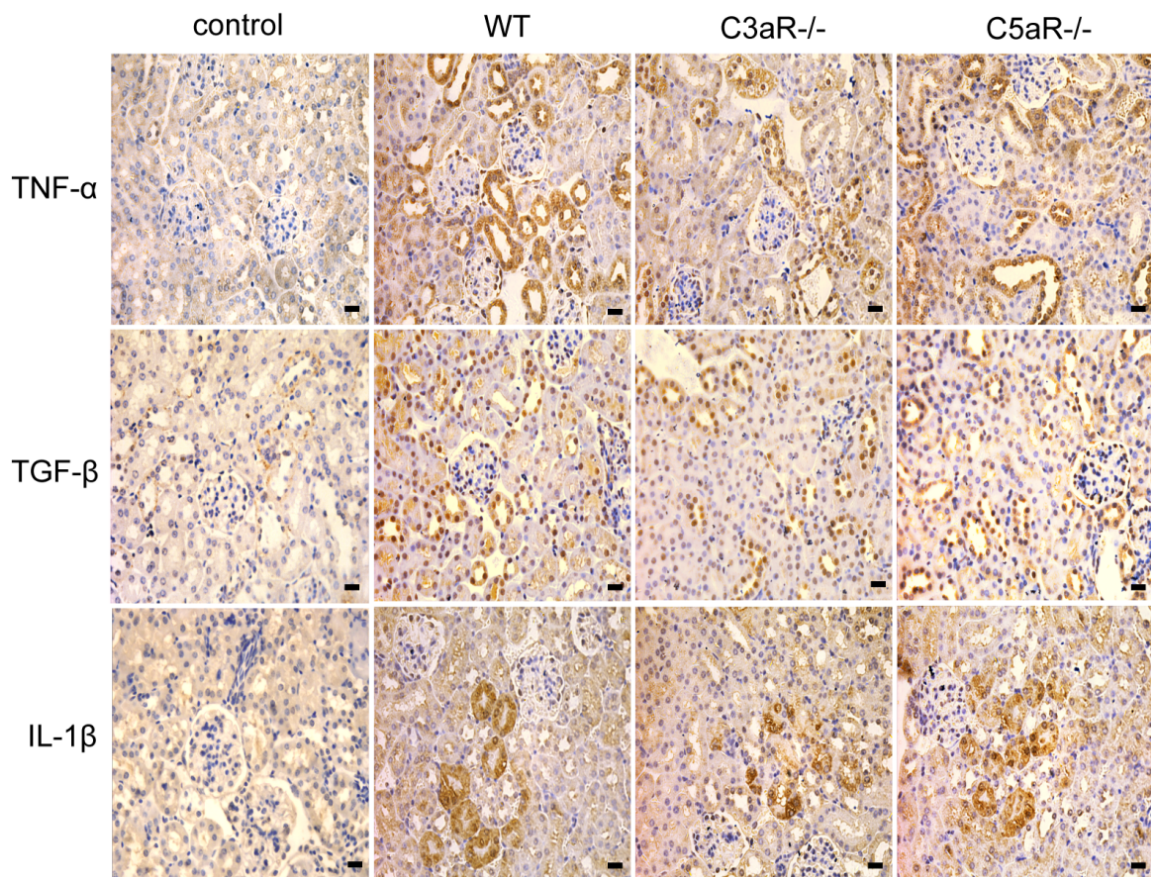


Fig. 4. Cytokine and chemokine gene expression in the renal tissues of the following four groups of mice: negative controls and WT, C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> IgAN mice ( $n = 7$  for each group). RT-qPCR was used to quantify the mRNA expression levels of (a) TNF- $\alpha$ , (b) TGF- $\beta$ , (c) IL-1 $\beta$ , (d) IL-6 and (e) MCP-1 relative to GAPDH. Data are expressed as the mean  $\pm$  SD. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  versus negative control.





Supplementary Figure S1. Representative images of immunohistochemical staining for TNF- $\alpha$ , TGF- $\beta$  and IL-1 $\beta$  in the kidney sections of WT, C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> IgAN mice and negative controls. C3aR and C5aR deficiency reduced renal TNF- $\alpha$ , TGF- $\beta$  and IL-1 $\beta$  expression compared to WT mice. Scale bars represent 100  $\mu$ m.